IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the application of: KHOWALA *ET AL.*	Group Art Unit: 1651
Serial No.: 9/773,365	Examiner: Irene Max
Filed: January 1, 2001	
For: A method of enhancing cellobiase activity of the novel strain Termitomyces clypeatus using 2-Deoxy-D-Glucose as Glycosylation inhibitor	Attorney Docket No: 8920-000005

To,
The Assistant Commissioner for Patents
Washington, D.C. 20231

Declaration Under 37 C.F.R. § 1.132

I, Suman Khowala, age 49 years, residing at, Institute of Chemical Biology (IICB), Kolkata, INDIA, and a citizen of India, do hereby state as under.

I am a Scientist at, Institute of Chemical Biology (IICB), Kolkata, INDIA. I graduated in the year 1974 from Calcutta University, Kolkota-700073, India. I completed my Master's Degree from Calcutta University, Kolkota-700073, India in the year 1976. Subsequently, I completed my doctoral degree in Biochemistry from Calcutta University, Kolkota-700073, India, in the Institute of Chemical Biology, Kolkata, India. After completing my doctoral degree, I took up my first assignment as a post-doctoral scientist with the present Institute i.e Institute of Chemical Biology, Kolkota-700073, India. After that, in 1990, I joined the same Institute, a constituent institution of the Council of Scientific and Industrial Research, India, as Scientist Gr.IV (1), where I continue to work in Biotechnology Group on the Regulation of Protein Export for last fourteen years. Presently, I am working as Scientist Group IV (3) of this institute (since Year 2000).

One of the projects undertaken by IICB, Kolkata, INDIA is "*A method of enhancing cellobiase activity of the novel strain Termitomyces clypeatus using 2-Deoxy-D-Glucose*

as Glycosylation inhibitor" This project was undertaken in the year 1997-1998. The scientists involved in the study were myself and Suman Mukherjee. I am the main scientist (project leader) in this study. I am aware of US patent application No. 9/773,365 filed in respect of this project. I am also aware and familiar with all the office actions, objections of the Examiner and the references cited by the Examiner. Therefore, I am completely and fully aware of all the facts relating to this project as well as the present patent application.

I wish to state at the outset that the challenge in the objective set out by the inventors is to develop a method of enhancing the cellobiase activity using 2-Deoxy-D-Glucose as a Glycosylator inhibitor. The organism used is fungus in *Termitomuces clypeatus* starin having Accession No. MTCC 5091, deposited at Institute of Microbial Technology (IMTECH), Chandigarh, India. This strain is open to public access and can be acquired/obtained from IMTECH, Chandigarh. The strain has been deposited with the Microbial Type Culture Collection & Gene Bank, Institute of Microbial Technology, Sector 39 A, Chandigarh 160 036, India, under the terms of the Budapest Treaty, and accorded the designation number MTCC 5091. The deposit will be maintained in the depository, which is a public depository, for a period of 30 years, or 5 years after the most recent request, or for the effective life of a patent, whichever is longer, and will be replaced if the deposit becomes depleted or non-viable during that period. Samples of the deposit will become available to the public and all restrictions imposed on access to the deposit will be removed upon grant of a patent on this application. A copy of the certificate is enclosed as requested by the Examiner.

The inventors wish to tell the Examiner that data of Table 1 showing cellobiase activity operates reveals the optimal concentration of sugar 2-deoxy-D-glucose needed for cellobiase activity. It is well known fact for enzymes that they function at an optimal level of substrates. Any increase or decrease in substrate concentrations alters the activity of ite enzyme molecules. In the present study the inventors observed that 2-deoxy-D-glucose has inhibitory affect on glycosylation process by functioning an optimal substrate for cellobiase enzyme. The inventors in the beginning of the experiment started with 1 mg/ml of the inhibitor 2-deoxy-D-glucose for studying the role of glycosylation in production of cellobiase from fungus *T.clypeatus*. It was observed during the experimentation that 2-deoxy-D-glucose regulated the specific activity and extracellular

secretion of cellobiase from the fungus, which has never been reported so far. It was observed that such regulation was based on a well known principle of Feed back inhibition (Ref: Principles of Biochemistry, Lehninger, Nelson, Cox, 1993, Worth Publishers, Inc.). Feed back inhibition regulates enzymes in such a manner that if their high amount of end product the regulatory activity is reduced. In the present study as shown in the table 1, it appears that an optimum concentration of 1 mg/ml of sugar, 2-deoxy-D-glucose is acceptable and produces the enzyme cellobiase at its maximum concentration. Any concentration increase of 2-deoxy-D-glucose does not enhance the enzyme activity rather it decreases its activity. In other words their excessive feedback inhibition by the cellobiase itself as shown in the Table 1, thereby inhibiting the growth of the fungus. In other words there is an optimal level of 2-deoxy-D-glucose is required for efficient and sufficient production of cellobiase and growth of the fungus.The inventors wish to state that the contention of the Examiner that 5mg/l of 2-deoxy-D-glucose gives less units of cellobiase is because of reasons mentioned above. The inventors have provided a revised table1 wherein various other concentrations of 2-deoxy-D-glucose which have been tried are also given. This very attributes of the 2-deoxy-D-glucose is due to feed back inhibition, which is a well known mechanism for enzymes and its related molecules.

Further, the inventors wish to inform the Examiner that addition of mannose was tested to see whether their any modulation (i.e. modulation in respect to increase or decrease of enzyme production) of the enzyme during the post-translation i.e. whether addition of mannose favor either growth of the fungus or modulate the extent of post-translational modification of the enzyme cellobiase, thereby modulating the activity or transport of cellobiase into extracellular medium. It was therefore observed with this study as shown by the data in the Table 2 (as given in the specification), that the enzyme, cellobiase translocation is affected by the mannose presence. Mannose as a sugar actually not only modulates the enzyme function but also facilitates the transportation of cellobiase from and through the cell membrane into the medium. It is also well known fact in literature that some proteins undergo posttranslational modifications thereby making more bioactive (Ref: Principles of Biochemistry, Lehninger, Nelson, Cox, 1993, Worth Publishers, Inc.). This very example has been undertaken to establish this fact for the enzyme cellobiase, which has never been reported thus far.

Table 1.Cellobiase activity in presence of glycosylation inhibitors

Samples	Cellobiase activity(units/ml)
Control	1.044
Control + Tunicamycin (10mg/ml)	1.2075
Control + 1-deoxynojirimycin (80mM)	1.4085
Control + 2-deoxy-D-glucose (0.05mg/ml) **Control + 2-deoxy-D-glucose (0.1mg/ml)**	2.236 **15.032**
Control + 2-deoxy-D-glucose (1mg/ml) **Control + 2-deoxy-D-glucose (2mg/ml)**	50.097 **12.501**
Control + 2-deoxy-D-glucose (5mg/ml)	0.081
Control + glucono-lactone (2mg/ml)	6.1820

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title XVIII of the United States Code and that willful false statements may jeopardize the validity of this Application for Patent or any patent issuing thereon.

Dated: June 25, 2004

Place: Indian Institute of Chemical Biology, Kolkata, INDIA

Dr. SUMAN KHOWALA